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Ras-Directed N-Glycoproteins Are Novel Early Biomarkers for Tumorigenesis and Malignant Transformation and Therapeutic Targets of Neurofibromatosis Type 1

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**14. ABSTRACT**
Neurofibromatosis type I is the most common familial cancer predisposition syndrome, the hallmark is the formation of neurofibromas, some of which will develop MPNSTs. In this report, glycoproteins are highly expressed in MPNST cell lines, MPNST clinical specimens, colon and pancreatic cancer cell lines with *Kras* mutations. Inhibition of protein glycosylation significantly inhibits the proliferation, migration and invasion of MPNST cell lines and inhibits the glycosylation and phosphorylation of tyrosine kinase receptors. Active Ras regulates MGAT5B expression and knockdown of MGAT5B significantly inhibits the phosphorylation of general tyrosine kinase receptors and their intracellular signaling. AKT interacts with and phosphorylates Ser192 in MGAT5B in MPNST cells. Moreover, wild type MGAT5B protein locates in the Golgi apparatus whereas mutated MGAT5B$^{S192A}$ protein retains in the ER and cytoplasm to inhibit MPNST lung tumor metastasis. In addition, PI3 kinase inhibition prevents MGAT5B trafficking from the cytoplasm to the Golgi apparatus to attenuate MGAT5B-mediated glycosylation and phosphorylation of kinase receptors. Targeting MGAT5-mediated glycosylation of general kinase receptors may provide a novel therapeutic approach for the treatment of MPNSTs and Ras-related malignancies.

**15. SUBJECT TERMS**
Neurofibromatosis type I, malignant peripheral nerve sheath tumors, Ras, MGAT5B, AKT, tyrosine kinase receptors, glycosylation, phosphorylation, tumorigenesis and metastasis.

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**Introduction……..…..**

Neurofibromatosis type I (NF1) is a dominantly inherited disease affecting 1 in every 2,500 to 3,000 individuals, representing the most common familial cancer predisposition syndrome[1]. It is a progressive condition with variable complications occurring over the time course of the disease. The hallmark of clinical manifestation of NF1 is the development of multiple neurofibromas[2], which are highly heterotypic benign tumors of peripheral nerve sheath mainly composed of immature Schwann cells, fibroblasts, perineurial and inflammatory matrix[3]. NF1 patients are also at high risk for the development of certain malignancies such as pheochromocytomas, childhood myeloid leukemias, neuroblastomas, rhabdomyosarcomas, and malignant peripheral nerve sheath tumors (MPNSTs)[4-6]. Once progressing to MPNSTs, although the resection is possible, most patients will eventually relapse locally or systemically. Because of the potential involvement of underlying nerves and blood vessels, surgical removal of tumors is not always an option[7]. Furthermore, once removed, the lesions have a tendency to regrow. There is no effective treatment for NF1, nor effective approaches for predicting or preventing the occurrence of devastating complications. Thus, to discover novel biomarkers to predict and develop agents for preventing or reversing the tumorigenesis and malignant transformation of NF1 are critically needed.

**Body…**……..

Although the specific cell of origin of neurofibromas and MPNSTs is uncertain, the predominant lesion associated with NF1 consists primarily of Schwann cells (60-80%)[8; 9]. Biallelic *Nf1* mutations have been detected in neurofibroma and MPNSTs with NF1[10; 11]. Schwann cells in neurofibroma and MPNST have been shown to possess abnormal properties, including increased invasiveness and induction of angiogenesis[12]. Schwann cells are peripheral nerve glia originating from migrating neural crest stem cells and recent studies have shown that NF1-related malignant astrocytomas originate from neural stem cells, raising the possibility that Schwann cell precursors (progenitors) undergoing *Nf1* loss of heterozygosity (LOH) during embryogenesis might be the neurofibroma-initiating cells[13]. Moreover, several NF1 mouse models have been generated to identify the Schwann cell progenitors as the 'cells of origin' of neurofibromas and MPNSTs[14; 15]. Schwann cell progenitors differentiate by late gestation and do not persist in the adult peripheral nervous system. However, Schwann cell progenitors persist or Schwann cells dedifferentiate into progenitor-like cells in neurofibromas in NF1[16]. Much evidence indicated that the loss of the *Nf1* gene in NF1 plays an important role in the initiation of tumorigenesis of neurofibromas and that all of the known NF1 phenotypes result from the inheritance or appearance of a mutant allele of the *Nf1* gene[17]. The *Nf1* gene product, neurofibromin,

functions as a GTPase activation protein (GAP), a negative regulator of the cellular Ras kinase[18]. It has been reported that the levels of activated Ras-GTP due to the loss of neurofibromin in NF1 plexiform neurofibromas and neurogenic sarcomas were approximately 5 and 15 times higher respectively, than the levels present in non-NF1 schwannomas, supporting the hypothesis that an aberrant Ras signaling pathway is the initial event in the tumorigenesis of NF1[19; 20]. However, the molecular mechanisms of the mutational inactivation of *Nf*1 resulting in hyperactive Ras that leads to alterations in uncontrolled growth and dedifferentiation of Schwann cells have not been elucidated in neurofibromas and MPNSTs.

One of the most important characteristics of transformed cells is an increase in N-glycosylation of cell surface proteins, known as the 'Warren phenomenon[21]. The appropriate and accurate modification of sugar or glycan to proteins mainly depends on the action of highly specific and precisely located enzymes known as glycosyltransferases and glycosidases in different tissue and cells[22]. It has been reported that activated Ras directs N-glycosylation in transformed cells and that these changes are most readily monitored by the analysis of complex-type N-glycosylation. For example, stable transfectants of NIH3T3 cells with activated *c-H-ras, c-K-ras or N-ras* are tumorigenic in nude mice and display the alterations in size-distribution of cell surface glycopeptides patterns which are highly correlated with invasiveness and metastasis[23]. Transient expression of activated *Ras* or overexpression of wild type *Ras* in NIH3T3 cells resulted in the significant differences in cell surface glycoproteins shortly after transfection and was independent of morphological transformation. It has been further reported that Ras differentially activates some glycosyltransferases to modify specific molecules involved in the malignant transformation. This is supported by the facts that the activities of the branching N-acetylglucosaminyltransferase III and V were elevated 2- to 2.5- fold whereas N-acetylglucosaminyltransferase I and II were unaltered, suggesting the formation of increased amounts of bisected glycans and structures carrying a Gal β1-GlcNAc β1-6Man-branch. The activities of the elongating β4-galactosyltransferase and β3-N-acetylglucosaminyltransferase were increased 5- to 7-fold in transformed cells, indicating a strongly enhanced capacity to synthesize polylactosaminoglycan chains[24; 25]. Moreover, Ras-directed N-linked carbohydrate modification on cell surface components and subsequent acquisition of invasiveness apparently precedes the morphological transformation[26]. Cancer-specific oligosaccharides in the serum of patients with pancreatic cancer have been determined to be useful biomarkers for pancreatic cancer, suggesting the possibility Ras-directed glycans present on the tumor cell surface may release and circulate in peripheral blood stream of patients[27], which may be the early diagnostic biomarkers for tumorigenesis and transformation of NF1.

Schwann cell progenitors are tumor-initiating cells of neurofibromas and MPNSTs and growth factor signaling is broadly implicated in the maintenance of the progenitor population[9; 28]. Growth factor receptors depend mainly on the glycosylation for stabilization, maturation, transportation to the cell surface, phosphorylation and activation. Neurofibromas and MPNSTs contain basic fibroblast growth factors, platelet-derived growth factors, insulin-like growth factor 2, neurogulin, as well as unidentified heparin-binding growth factors[29]. Mature Schwann cells normally lack the expression of EGFR and c-MET, however, Schwann cells from human neurofibromas and MPNST cells with NF1 express high levels of EGFR and c-MET[30]. Schwann cell progenitors from *Nf1+/-* and *Nf1-/-*mouse embryos expressed high level of EGFR and c-MET, as well as other growth factor receptors such as erbB2 and erbB3[31; 32]. In addition, both EGFR and c-MET have been specifically implicated in central or peripheral nervous system progenitor expansion and associated with the tumorigenesis in *Nf1+/-:p53+/-* mouse tumor model[33]. Thus, we hypothesize that these mitogenic cytokines that are not present in normal peripheral nervous system would act on *Nf*1+/- and *Nf*1-/- Schwann cells in NF1 to maintain elevated Ras signaling and to directly mediate downstream intracellular signaling through their cognate receptors, which synergistically promote population of Schwann cell progenitors that have lost the inhibitory signaling normally provided by axons in neurofibromas and MPNSTs in NF1.

Glycosylation is a common synthetic step for many transmembrane receptor families that are targets for cancer therapy including EGFR, c-MET, IGF-RI, c-KIT, RET and VEGFR. However, recent studies have demonstrated cancer cell resistance to targeted therapies for single or multiple tyrosine kinase receptors, which is caused by the activation of parallel and compensatory receptor-mediated intracellular signaling cascades[34]. Thus, inhibiting most, if not all, of growth factor receptors that are deregulated in Schwann cell progenitors of NF1 may be of therapeutic benefit. We propose to selectively inhibit the glycosylation of growth factor receptors that are deregulated by hyperactive Ras in Schwann cell progenitors to open a new preventive and therapeutic window for NF1.

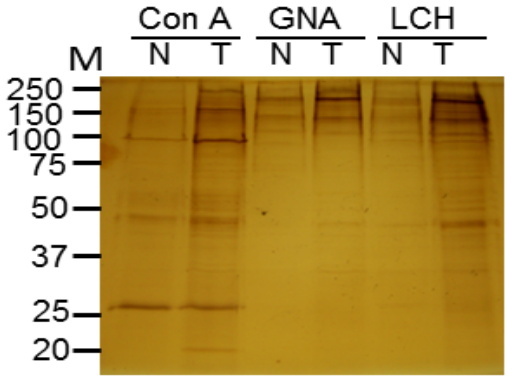

Figure1. N-glycoproteins from 1x10$^7$ normal Schwann cells (N) or MPNST cells (T) were fractionated by N-glycoprotein fractionation kit from Qiagen and subject to SDS-PAGE followed by silver staining

**Key Research Accomplishments and reportable outcomes.**

***N-Glycoproteins are highly expressed in MPNST cells and 2-DG is a potential glycosylation inhibitor.***

N-linked and O-linked oligosaccharide variants on glycoproteins can lead to alterations in protein activity or function that may manifest themselves as overt disease. Most plasma membrane and secreted proteins are glycosylated. In order to detect aberrant expression of N-glycoproteins in MPNST cell lines, we used the Qproteome Mannose Glycoprotein Kit (Qiagen) to detect the expression levels of N-glycoproteins in normal Schwann cells (NSCs) and MPNST cell lines. This kit containing three lectins to bind N-glycoproteins: Con A (concanavalin A), LCH (lentil lectin) and GNA (snowdrop lectin). Branched a-mannosidic structures, high-mannose type, hybrid type and biantennary complex type N-glycans binding proteins were observed in lectin ConA; fucosylated core region of bi and triantennary complex type N-glycans binding proteins were observed in lectin LCH and $\alpha$1-3 and $\alpha$1-6 linked high mannose structures glycans binding proteins were observed in lectin GNA.

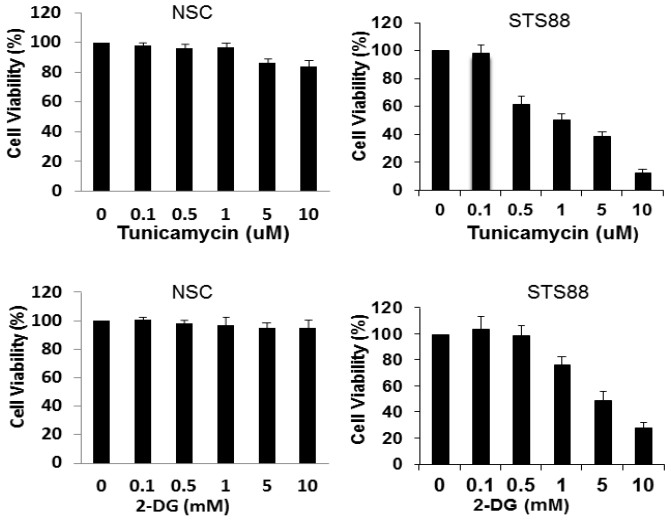


Figure2. Normal Schwann cells and MPNST cells were treated with Tunicamycin or 2-DG at various doses. Cell viability (%) was determined by MTS assay, data represent 72 hours treatment.

It has been reported that loss of *Nf1* leads to the activation of Ras which upregulates the expression of glycoproteins[18;35]. Using Qproteome Mannose Glycoprotein Kit, we detected the expression levels of N-glycoproteins in NSCs and MPNST cell lines. Compared with human NSCs (N), MPNST cells (T) express high levels of N-glycoproteins and display different N-glycoprotein signatures (Figure1).

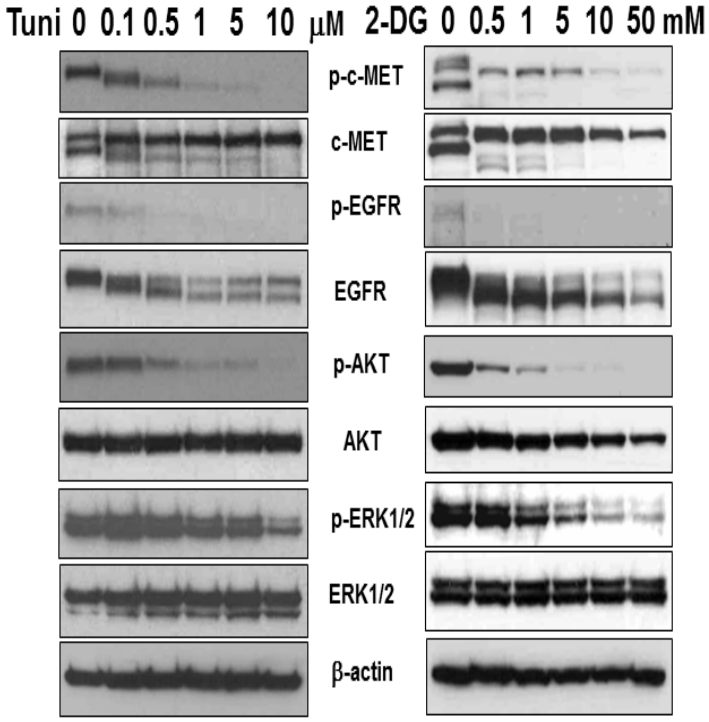


Figure3. MPNST cells were treated with Tunicamycin or 2-DG for 24 hours respectively, cell lysates were used to blot with indicated antibodies. The impaired glycosylation was demonstrated by the decreased molecular size and fast shift mobility; impaired maturation of c-MET was visualized by intact premature chain accumulation (upper band).

***The inhibition of protein glycosylation impaired the phosphorylation of kinase receptors and their downstream signaling.***

Because proteins travelling to the Golgi apparatus for the consequent steps of glycosylation must be bound by mannose-6-phosphate in order to attach itself to the mannose-6-phosphate receptor and because of the structural similarity between mannose and 2-Deoxy-D-Glucose (2-DG), we hypothesized that 2-DG may be a competitor of mannose for inhibiting protein glycosylation processes. To investigate whether the glycosylation status of kinase receptors affects phosphorylation status of kinase receptors, we selected commercial available glycosylation inhibitor Tunicamycin and 2-DG as two glycosylation inhibitors. We treated MPNST cell lines with these two inhibitors respectively in different doses for 24, 48 and 72 hours, we found that both Tunicamycin and 2-DG inhibited the proliferation of MPNST cell lines but had no inhibitory effect on NSCs (Figure 2).

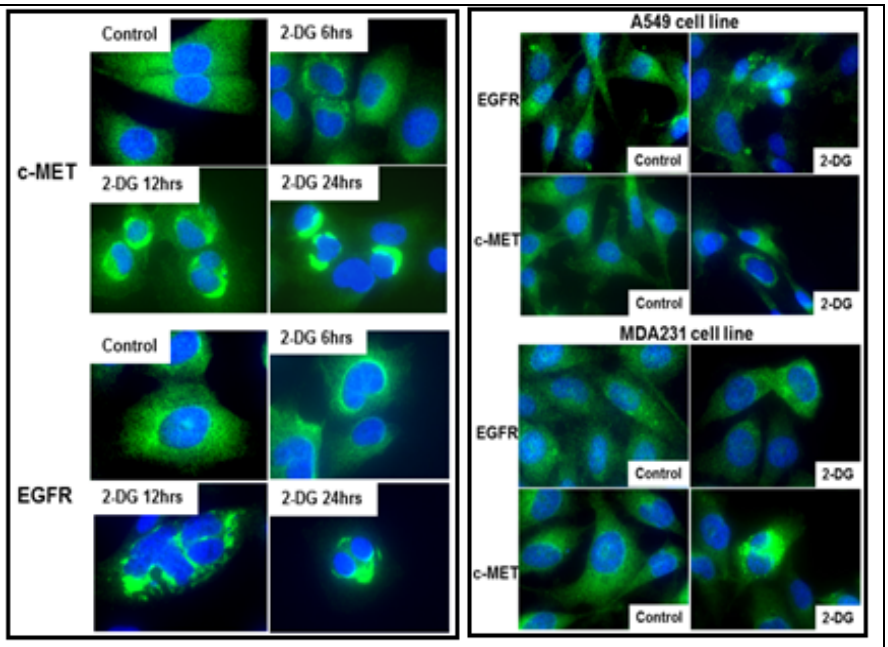


Figure4. 2-DG inhibited the transportation of receptors from the cytoplasm to the cell surface and retained receptors in the ER and Golgi apparatus.

Both Tunicamycin and 2-DG inhibited the glycosylation (faster shift) and phosphorylation of EGFR and c-MET and impaired MEK-ERK1/2 and PI3K-AKT intracellular signaling in a dose dependent manner (Figure 3). In addition, the precursor of c-MET is proteolytically cleaved at a furin site to yield a highly glycosylated extracellular α-subunit and a transmembrane β-subunit, which are linked together by a disulfide bridge[36]. We found that 2-DG inhibited the glycosylation and maturation of c-MET as demonstrated by accumulation of precursor, and c-MET was failed to cleave into one mature glycosylated extracellular α-subunit (Figure 3) and a transmembrane β-subunit. We also detected poorly glycosylated EGFR (small EGFR band with fast shifting) in MPNST

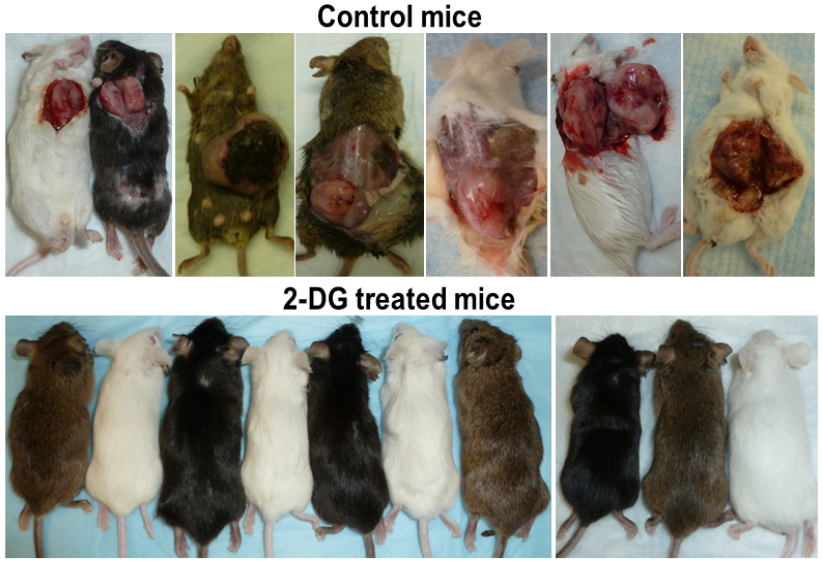


Figure5. *Nf1+/-;p53+/-* mice developed tumors (top) whereas 2-DG prevented tumorigenesis in *Nf1+/-;p53+/-* mice (bottom).

Cells (Figure 3). Both Tunicamycin and 2-DG inhibited the phosphorylation of AKT and ERK1/2 in a dose dependent manner (Figure 3). Moreover, 2-DG inhibited the transportation of receptors from the cytoplasm to the cell surface and retained receptors in the ER and Golgi apparatus (Figure 4 left panel). 2-DG also induced the accumulation of c-MET and EGFR in the ER and Golgi apparatus in lung and breast cancer cell lines (Figure 4 right panel). To date, this is the first time for us to detect 2-DG inhibiting the glycosylation and phosphorylation of kinase receptors. These data suggest that inhibiting glycosylation of kinase receptors attenuates their phosphorylation and activation, and blocks their intracellular signaling

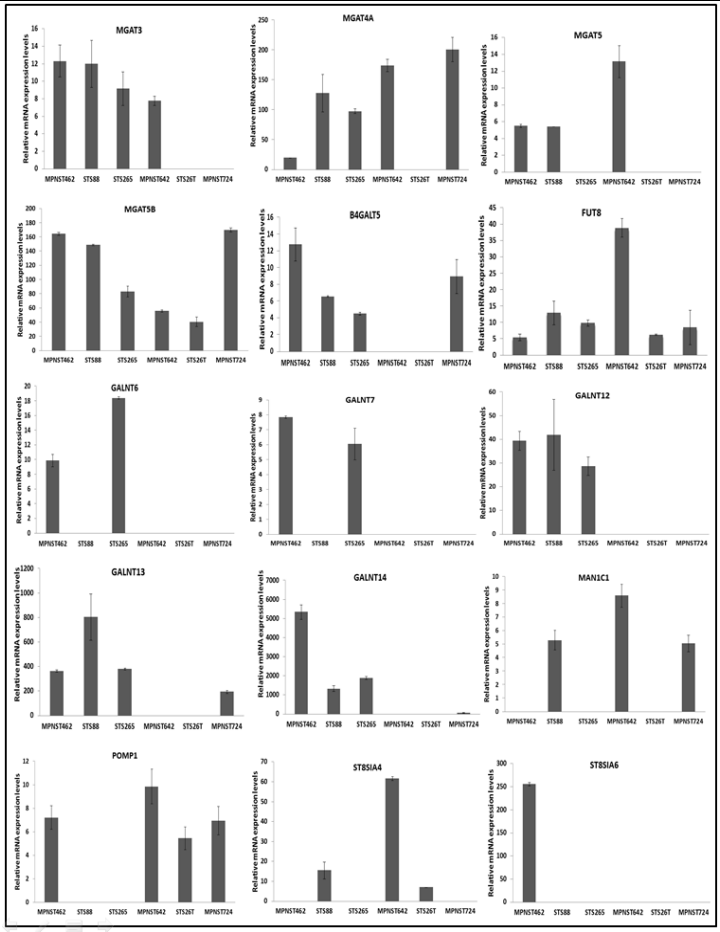


Figure6. Glycosyltransferase mRNA exprssion levels in different MPNST cell lines.

***2-DG prevented tumor development of Nf1+/-; p53+/- mice.***

It has been reported that 85% of *NF1+/-; p53+/-* mice will develop tumors, most of them are MPNSTs in the age of 15 or long weeks[37]. When *NF1+/-; p53+/-* mice were genetically identified and grew up to 6 weeks, we randomly separate these mice into two groups: control group containing 30 *NF1+/-;p53+/-* mice were treated with 25mg/kg of glucose per day and treatment group containing 30 *NF1+/-;p53+/-* mice were treated with 25mg/kg of 2-DG per day by oral gavage. We found that two mice in control group and three mice in treatment group died during the treatment process. 20 of 28 mice (71.42%) in the control group and 2 of 27 mice (7.4%) in the 2-DG treated group developed tumors (Figure 5, partial data shown because of limited space), suggesting 2-DG has a potential ability to prevent the tumor development in *NF1+/-;p53+/-* mice.

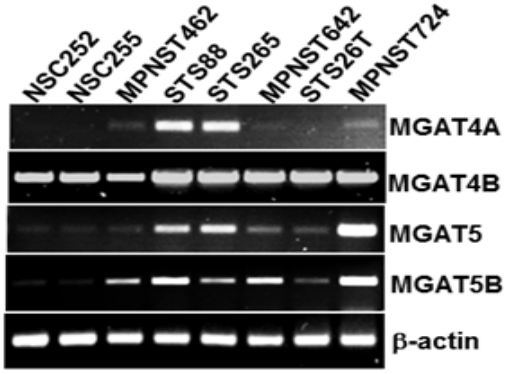


Figure7. RT-PCR to detect the mRNA expression of MGAT4A,MGAT4B,MGAT5 and MGAT5B in MPNST cell lines

***Glycosyltransferases are highly expressed in MPNSTs.***

The appropriate and accurate modification of sugar or glycan to proteins mainly depends on the action of highly specific and precisely located enzymes known as glycosyltransferases and glycosidases in different tissue and cells. To detect the expression of glycosyltransferases and glycosides in NSCs and MPNST cell lines, the Human Glycosylation RT² Profiler™ PCR Array (Qiagen) has been used to profile the expression of 84 key genes encoding enzymes that add glycans (glycosyltransferases) to proteins or remove glycans (glycosidases) from glycoproteins. Compared with NSCs, we did not find any change in glycosidase mRNA expression levels between NSCs and MPNST cell lines. However, multiple glycosyltransferases such as MGAT3, MGAT4A, MGAT5, MGAT5B, B4GALT5, FUT8, GALNT6, GALNT7, GALNT12, GALNT13, GALNT14, MAN1C1, POMT1 ST8SIA4 and ST8SIA6 were overexpressed at different levels in different MPNST cell lines. Especially MGAT5B and FUT8 mRNA was highly expressed in all MPNST cell lines (Figure 6). The mRNA expression levels of MGAT4A, MGAT4B, MGAT5 and MGAT5B were confirmed in NSCs and MPNST cell lines by RT-PCR (Figure 7).

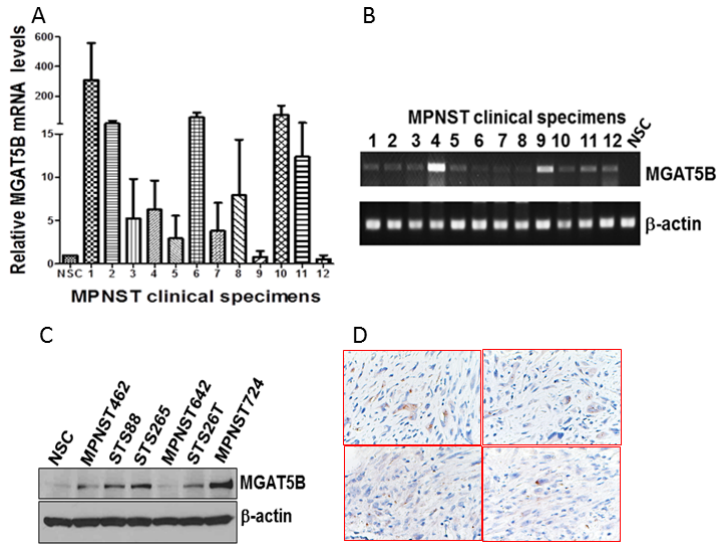


Figure8. A: (qPCR) and B: (RT-PCR) detect MGAT5B mRNA expression in normal Schwann cells and MPNST clinical specimens. C: MGAT5B protein expression in normal Schwann cells and MPNST cell lines. D: MGAT5B protein preferentially locates in the Golgi apparatus of the cells in MPNST clinical specimens.

***The Expression of MGAT5B correlated with hyperactive Ras in MPNST and Ras-related malignancies.***

Because MGAT5B is highly expressed in all MPNST cell lines, we focused on its role in MPNST development and metastasis. We detect MGAT5B mRNA expression levels by qPCR and RT-PCR in NSCs and MPNST clinical specimens, we found that most of MPNST clinical specimens expression high levels of MGAT5B (Figure 8 A&B). MGAT5B protein expression was also confirmed by western blot in NSCs and MPNST cell lines (Figure 8C), indicating that NCSs express less whereas MPNST cell lines express

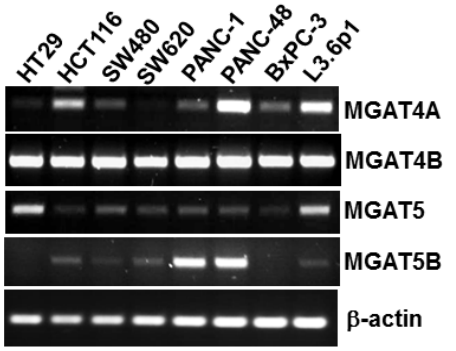


Figure9. MGAT5B preferentially expresses in colon (HCT116, SW480, SW620) and pancreatic (PANC1, PANC-48 and L3.6p1) cancer lines with Kras mutation, but not in colon (HT29) and pancreatic (BxPC-3) cancer cell lines without Kras mutations

high level of MGAT5B protein. We also detected that MGAT5B protein is located in the Golgi apparatus in tumor cells by immunohistochemical staining in MPNST clinical specimens (Figure 8D).

We also found that MGAT5B preferentially express in colon and pancreatic cancer lines with *Kras* mutations but has no or low levels of expression in colon and pancreatic cancer lines without *Kras* mutation (Figure9). MGAT5B expression was also detected in primary colon cancer cells and metastatic colon cancer cells in liver (Figure 10). These data suggested that MGAT5B expression is regulated by Ras kinase activity. Patients with NF1 have a 10% lifetime risk of developing MPNSTs and loss of *Nf1*[38] gene leading to the hyperactive Ras is the key step to develop these malignancies. It has been reported that Ras upregulates the expression of glycoproteins but molecular mechanism has not been elucidated. In our experiments, we found that active Ras is highly expressed in MPNST cell lines (Figure 11A) and MGAT5B mRNA expression levels correlated with active RAS in MPNST cell lines (Figure 11B). We also found that knockdown of *Nf1* or overexpression of *K-ras*$^{G12D}$ significantly upregulated MGAT5B mRNA expression levels in STS26T cells (Figure 11C&D). These data suggest that RAS regulates MGAT5B mRNA expression in MPNST and Ras-related malignancies.

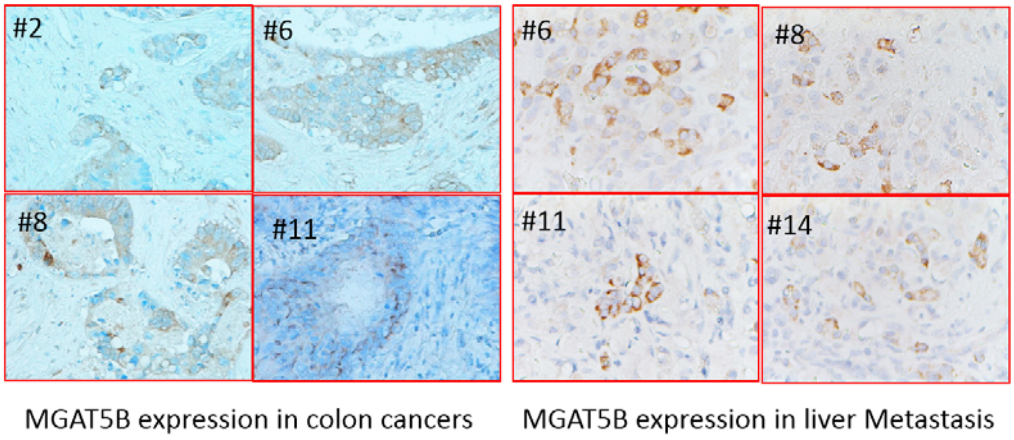


Figure10. Left: MGAT5B expression in colon cancer clinical specimens; Right: MGAT5B expression in metastatic colon cancel cells in the liver.

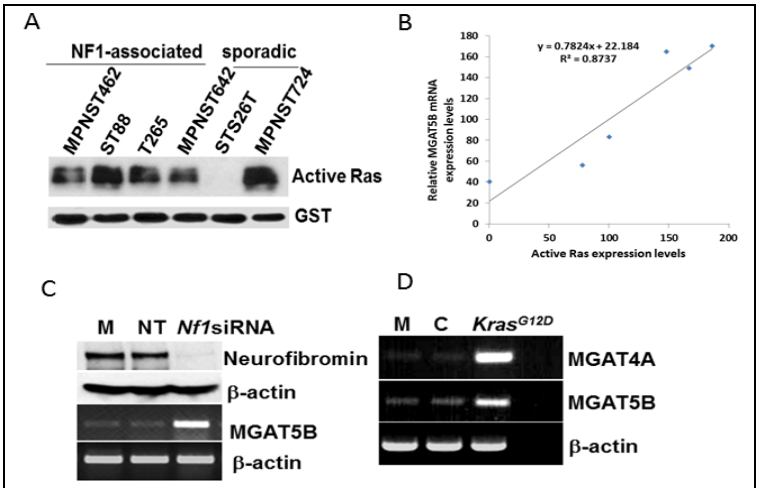


Figure11. A: Active Ras in MPNST cell lines. B:Ras activity is correlated with MGAT5B expression levels. C: knockdown of *Nf1* upregulated MGAT5B mRNA expression. D: overexpression of KrasG12D upregulated MGAT4A and MGAT5B expression.

***MAGT5B is a potential AKT phosphorylation protein.***

Since MGAT5B is preferentially located in the Golgi apparatus, suggesting that posttranslational modification such as phosphorylation may promote MGAT5B transportation from the cytoplasm into Golgi apparatus. Motif Scan (www.scansite.mit.edu) graphic results indicated Ser192 (RARWTSD) in

MGAT5B is the potential AKT phosphorylation site (Table 1). Because AKT is highly activated in MPNSTs, we hypothesize that AKT may phosphorylates ser192 in MGAT5B to promote its trafficking from the cytoplasm into the Golgi apparatus to promote its glycosyltransferase activity. To investigate this, MGAT5B full length cDNA was cloned into pEGFPN1 to generate pEGFPN1-MAGT5B, and site-directed mutagenesis (Stratagene) was used to mutate Ser 192 into Alan 192 (S192A) to generate pEGFPN1-MGAT5B$^{S192A}$ (mutated). Two plasmid constructs were transfected into STS26T cells, the localization of GFP-tagged MGAT5B or GFP-tagged MGAT5B$^{S192A}$ was observed under the fluorescence microscope, we found that wild type MGAT5B protein was preferentially located in Golgi apparatus while MGAT5B$^{S192A}$ (mutated) was distributed in the ER and cytoplasm (Figure 12), suggesting the modification of ser192 at MGAT5B may involve in MGAT5B trafficking. Because commercial available anti-MGAT5B antibody can nonspecifically recognize multiple protein bands by western blot, we made Myc-tagged MGAT5B construct (pcDNA3.1-MGAT5B-Myc plasmid) and transfected it into MPNST724 cells and whole cell lysates were immunoprecipitated with either an anti-AKT or anti-Myc antibody and blotted with anti-Myc or anti-AKT antibody respectively, we found MGAT5B-Myc protein in AKT immunocomplexes and AKT protein in the MGAT5B-Myc immunocomplexes (Figure13A), suggesting AKT interacts with MGAT5B in living cells.

| Akt Kinase | | | Gene Card AKT1 | |
|---|---|---|---|---|
| Site | Score | Percentile | Sequence | SA |
| S192 | 0.5595 | 0.590 % | WMRARWTSDPCYAFF | 0.645 |

Table1. Motif scan (www.scansite.mit.edu) indicates that S192 in MGAT5B is an AKT phosphorylation site.

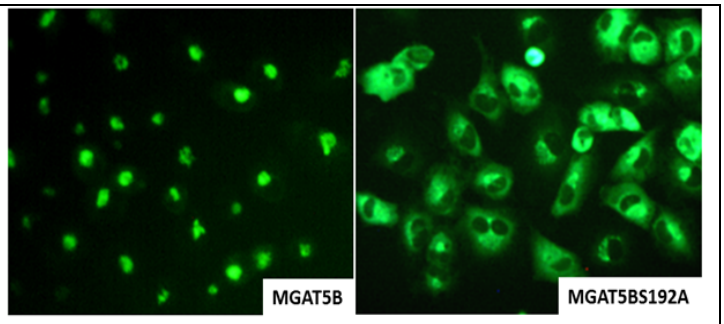


Figure12: wild type MGAT5B is located in the Golgi apparatus whereas mutated MGAT5Bs192A is retained in the cytoplasm.

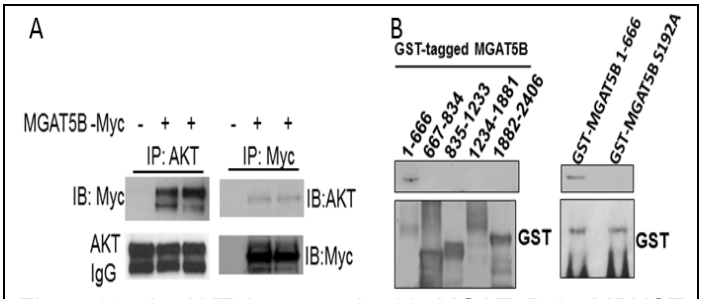


Figure13. A: AKT interacted with MGAT5B in MPNST cell lines. B: AKT phosphorylated S192 in MGAT5B.

To determine whether MGAT5B is an AKT phosphorylation protein, we cloned human MGAT5B cDNA fragments (nucleotide 1- 666, 666-834, 835-1233,1224-1881,1882-2406) into pGEX4T1 plasmid to generate GST-MGAT5B fusion proteins. We purified the GST-MGAT5B fusion proteins as AKT substrates, in the present of constitutively activated AKT1 (Invitrogen, cat#2999) and [gamma32P]ATP (Perkin Elmer), we found that GST-MGAT5B fusion protein containing S192 was phosphorylated in the presence of AKT1 whereas no phosphorylation was observed in GST-MGAT5B fusion protein with S192A mutation (Figure 13B), suggesting S192 in MGAT5B is an AKT

phosphorylation site.

***AKT kinase blockade inhibited glycosylation of kinase receptors and arrested MGAT5B in the cytoplasm.***

We treated MPNST cells with PI3K inhibitor Ly294002 in different doses for 24 hours, we found AKT kinase is inhibited (Figure 13A). We also detected the phosphorylation of c-MET and EGFR was inhibited in a dose dependent manner in MPNST cells (Figure 14A). We hypothesized that PI3K inhibitor-mediated phosphorylation of kinase receptors was induced by AKT-mediated MGAT5B glycosyltransferase activity inhibition. Furthermore, we treated MPNST cells expressing MGAT5B-GFP with control (DMSO) or PI3K inhibitor Ly294002 (Ly) for 24 hours and found that MGAT5B-GFP protein stayed in the Golgi apparatus in control group, when AKT kinase was inhibited by Ly294002, MGAT5B-GFP protein distributed in the ER and the cytoplasm (Figure14 B). These data suggested that AKT interacts with and phosphorylates MGAT5B to promote MGAT5B trafficking from the cytoplasm to the Golgi apparatus to promote glycosylation and phosphorylation of kinase receptors to drive tumor proliferation and metastasis.

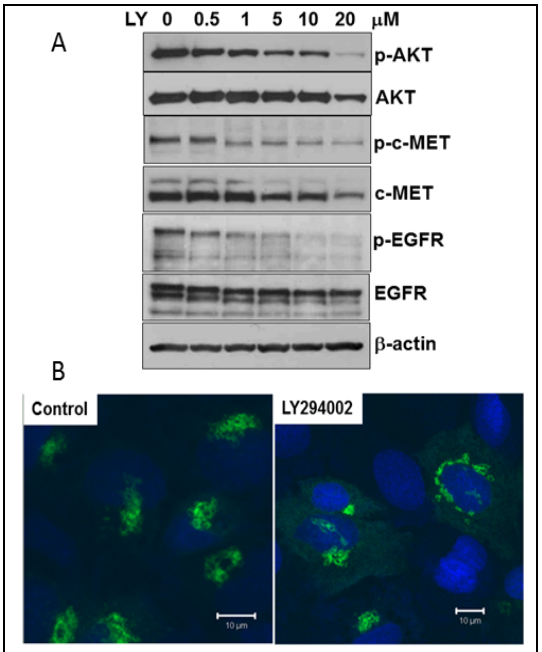


Figure14. A: AKT interacted withMGAT5B in MPNST cells. B: AKT phosphorylated S192 in MGAT5B.

| Table 2 Lung tumor metastasis of MPNST cell lines | | | |
|---|---|---|---|
| Cell lines | GFP | MGAT5B | MGAT5BS192A |
| Numbers of total mice | 6 | 6 | 6 |
| Numbers of mice with lung metastasis | 4 | 5 | 1 |
| Metastatic rate (%) | 66.7 | 83.3 | 16.7 |

**MGAT5B promoted MPNST tumor lung metastasis.**

We transfected pEGFPN1, pEGFPN1-MGAT5B or pEGFPN1-MGAT5BS192A plasmid respectively into STS26T cells to establish three stable cell lines. We injected these three cell lines separately into the tail veins of hairless SCID mice to observe the experimental lung tumor metastasis. After 8 weeks of breeding, mice were sacrificed, lung weights were measured and tumor micrometastasis in lung was measured under the microscope. We found 4 of 6 mice in GFP group, 5 of 6 mice in MGAT5B group showed experimental tumor lung metastasis while only I of 6 in MGAT5BS192A mice showed experimental tumor lung metastasis (Table 2 &Figure 15), suggesting MGAT5BS192A plays a dominant negative role and significantly inhibits *in vivo* experimental lung tumor metastasis.

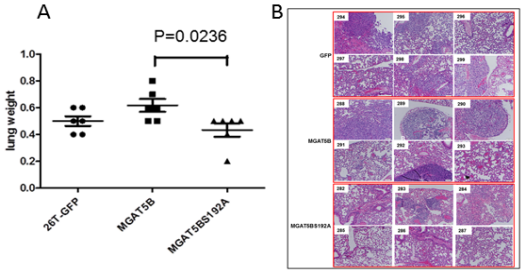


Figure15. A: mice lung weights. B: MPNST lung tumor metastasis, each group has 6 mice.

***MGAT5B mediated glycosylation and phosphorylation of kinase receptors***

To investigate the role of MGAT5B in mediating glycosylation and phosphorylation of kinase receptors, we knockdown MGAT5B in MPNST cell lines. Transient knockdown of MGAT5B (siRNA) blocked the phosphorylation of c-MET and EGFR (Figure 16A), inhibited the cell proliferation (Figure16B), impaired migration and invasion (Figure 16C), arrested cells in G2 phase (Figure16D) and induced apoptosis of MPNST cell lines (Figure16E). Stable knockdown of MGAT5B (shRNA) significantly inhibited MGAT5B mRNA expression levels (Figure 17A); arrested cells in G1 phase (Figure 17B). Stable knockdown of MGAT5B also blocked the phosphorylation of kinase receptors such as c-MET, c-Ret, FGFR, Tie, EGFR, AXL, EphR, EphA4, ROR, PDGFR, et al (Figure 18A). In addition, the morphology of MPNST cells normally is round or polygonal, with few cells showing elongations. However, when MGAT5B is knocked down, filamentous protrusions began to appear and cell shapes shrunk, suggesting shRNA knockdown of MGAT5B is associated with a marked alterations in cell morphology (Figure 18B). These data suggest that downregulation of MGAT5B significantly attenuates the phosphorylation of kinase receptors and their intracellular signaling and induce growth inhibition and cell differentiation.

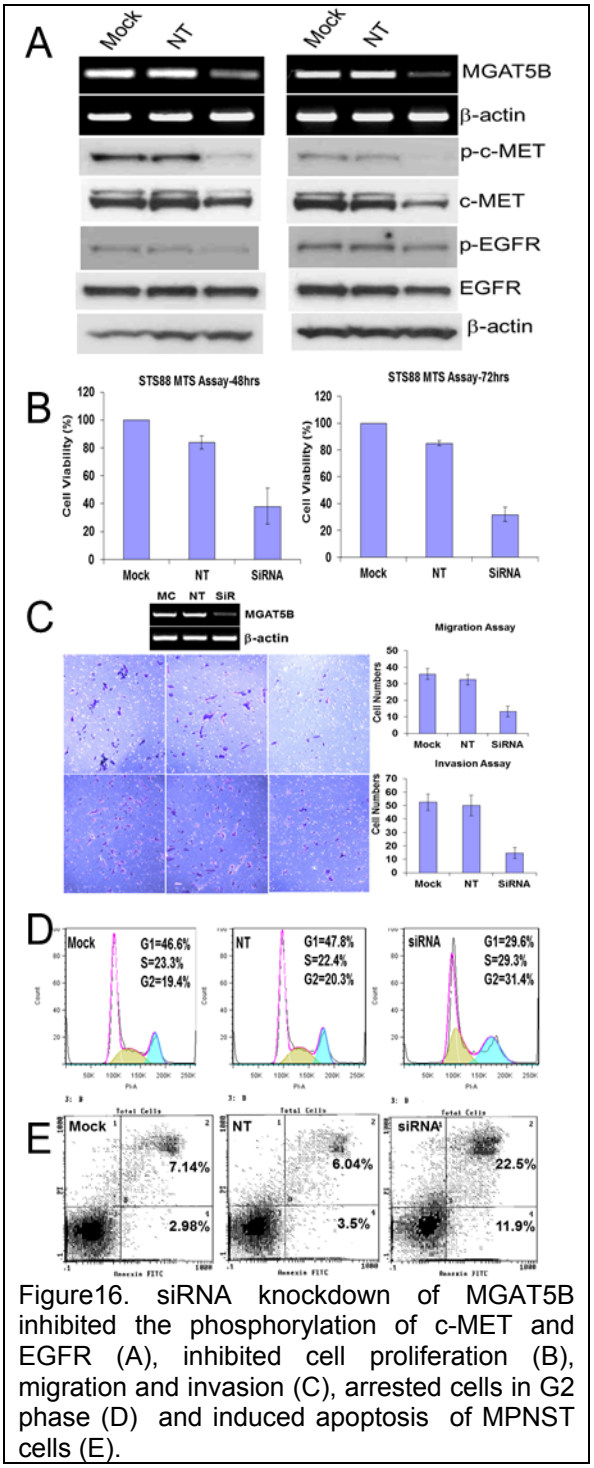


Figure16. siRNA knockdown of MGAT5B inhibited the phosphorylation of c-MET and EGFR (A), inhibited cell proliferation (B), migration and invasion (C), arrested cells in G2 phase (D) and induced apoptosis of MPNST cells (E).

**The ongoing experiments**

Tumorigenesis from Schwann cell progenitors: Mouse neural crest cells develop into Schwann cell precursors between E11 and E13 in mouse sciatic nerve, these cells will develop immature Schwann cells by E15 and mature Schwann cells by E18 (Jessen and Mirsky,2005). We have generated *Nf1:p53* animal models in which *Nf1+/-;p53+/-* mice developed tumors in postnatal 15 weeks, most of them are MPNSTs. Because *Nf1-/-* mice are embryonic lethality in 12 day gestation. We dissociated E12.5 dorsal root ganglion (DRG) from *Nf1-/-,p53-/-* embryos (dead embryos) to make single cell suspension and culture in the stem cell media. We

sorted the Schwann cell progenitors containing EGFR$^{+}$; P75$^{+}$ cells. These cells are neurofibroma-initiating cells and form tumors in nude mice. We utilized Flow cytometry to sort EGFR$^{+}$:P75$^{+}$ cells from postnatal sciatic nerve and culture in the stem cell media but we could not isolate EGFR$^{+}$:P75$^{+}$ cells by flow cytometry, we are not able to inject these cells into SCID mice to develop tumors**.**

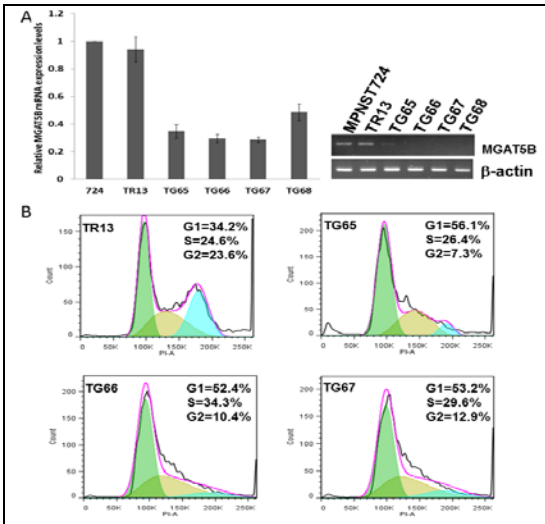


Figure17. siRNA knockdown of MGAT5B inhibited the phosphorylation of c-MET and EGFR (A), inhibited cell proliferation (B), migration and invasion (C), arrested cells in G2 phase (D) and induced apoptosis of MPNST cells (E).

**Conclusion……**

**…**

1. 1 N-glycosylation signatures indicated that MPNST cells express high level of N-glycosylation proteins compared with normal Schwann cells.
2. 2-DG, a competitor of mannose, is a novel glycosylation inhibitor, inhibiting the glycosylation and phosphorylation of tyrosine kinase receptors, and blocking the transportation of kinase receptors from Golgi apparatus to cell surface.
3. 2-DG prevents the tumorigenesis in *NF1+/-; p53+/-* mice.
4. MGAT5B, one of the glycosyltransferases, is highly expressed in MPNST cell lines and MPNST clinical specimens, which is correlated with hyperactive Ras kinase activity, and *Ras* upregulates the expression of MGAT5B in MPNST cell lines.

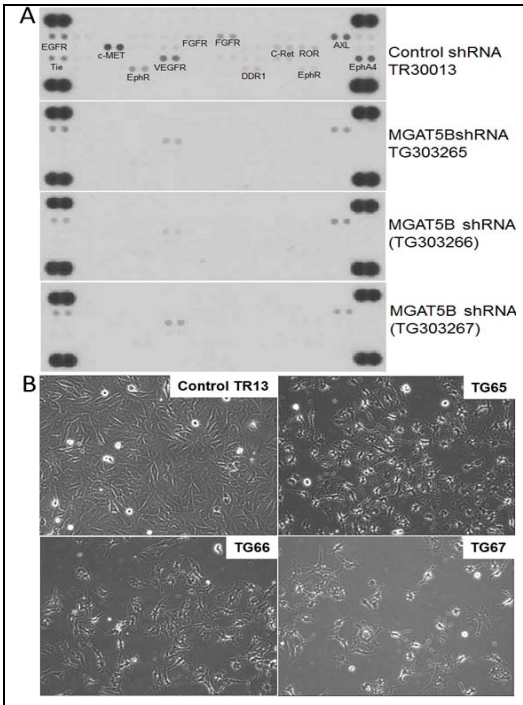


Figure18. A: siRNA knockdown of MGAT5B inhibited the phosphorylation of multiple receptor tyrosine kinases. B: siRNA knockdown of MGAT5B induced cell differentiation.

5. Knockdown of MGAT5B significantly inhibits the glycosylation and phosphorylation of general kinase receptors and receptor-mediated intracellular signaling, inhibits the migration and invasion of MPNST cell lines and phosphorylation of multiple receptor tyrosine kinases such as c-MET, c-Ret, FGFR, Tie, EGFR, AXL, EphR, EphA4, ROR, PDGFR, et al.
6. Ser192 in MGAT5B is phosphorylated by AKT and mutation of Ser192 in MGAT5B (MGAT5BS192A) blocks its transportation from the cytoplasm to Golgi apparatus and significantly inhibits experimental lung tumor metastasis.

**References…….**


1. Gerber, P.A., Antal, A.S., Neumann, N.J., Homey, B., Matuschek, C., Peiper, M., Budach, W., and Bolke, E. (2009). Neurofibromatosis. European journal of medical research 14, 102-105.
2. Elvsashagen, T., Solyga, V., Bakke, S.J., Heiberg, A., and Kerty, E. (2009). [Neurofibromatosis type 2 and auditory brainstem implantation]. Tidsskrift for den Norske laegeforening : tidsskrift for praktisk medicin, ny raekke 129, 1469-1473.
3. Krone, W., Jirikowski, G., Muhleck, O., Kling, H., and Gall, H. (1983). Cell culture studies on neurofibromatosis (von Recklinghausen). II. Occurrence of glial cells in primary cultures of peripheral neurofibromas. Human genetics 63, 247-251.
4. Knudson, A.G., Jr., and Meadows, A.T. (1976). Developmental genetics of neuroblastoma. Journal of the National Cancer Institute 57, 675-682.
5. Abell, M.R., Hart, W.R., and Olson, J.R. (1970). Tumors of the peripheral nervous system. Human pathology 1, 503-551.
6. Hope, D.G., and Mulvihill, J.J. (1981). Malignancy in neurofibromatosis. Advances in neurology 29, 33-56.
7. Gaudi, S., Mills, O., Goyette, E.F., and Morgan, M.B. (2011). Intravascular schwannoma. The American Journal of dermatopathology 33, 850-854.
8. Ferner, R.E., and O'Doherty, M.J. (2002). Neurofibroma and schwannoma. Current opinion in neurology 15, 679-684.
9. Carroll, S.L., and Ratner, N. (2008). How does the Schwann cell lineage form tumors in NF1? Glia 56, 1590-1605.
10. Fang, Y., Elahi, A., Denley, R.C., Rao, P.H., Brennan, M.F., and Jhanwar, S.C. (2009). Molecular characterization of permanent cell lines from primary, metastatic and recurrent malignant peripheral nerve sheath tumors (MPNST) with underlying neurofibromatosis-1. Anticancer research 29, 1255-1262.
11. Lothe, R.A., Smith-Sorensen, B., Hektoen, M., Stenwig, A.E., Mandahl, N., Saeter, G., and Mertens, F. (2001). Biallelic inactivation of TP53 rarely contributes to the development of malignant peripheral nerve sheath tumors. Genes, chromosomes & cancer 30, 202-206.
12. Torres, K.E., Zhu, Q.S., Bill, K., Lopez, G., Ghadimi, M.P., Xie, X., Young, E.D., Liu, J., Nguyen, T., Bolshakov, S., et al. (2011). Activated MET is a molecular prognosticator and potential therapeutic target for malignant peripheral nerve sheath tumors. Clinical cancer research : an official journal of the American Association for Cancer Research 17, 3943-3955.
13. Alcantara Llaguno, S., Chen, J., Kwon, C.H., Jackson, E.L., Li, Y., Burns, D.K., Alvarez-Buylla, A., and Parada, L.F. (2009). Malignant astrocytomas originate from neural stem/progenitor cells in a somatic tumor suppressor mouse model. Cancer cell 15, 45-56.
14. Roth, T.M., Ramamurthy, P., Ebisu, F., Lisak, R.P., Bealmear, B.M., and Barald, K.F. (2007). A mouse embryonic stem cell model of Schwann cell differentiation for studies of the role of neurofibromatosis type 1 in Schwann cell development and tumor formation. Glia 55, 1123-1133.
15. Kim, H.A., Ling, B., and Ratner, N. (1997). Nf1-deficient mouse Schwann cells are angiogenic and invasive and can be induced to hyperproliferate: reversion of some phenotypes by an inhibitor of farnesyl protein transferase. Molecular and cellular biology 17, 862-872.
16. Gottfried, O.N., Viskochil, D.H., and Couldwell, W.T. (2010). Neurofibromatosis Type 1 and tumorigenesis: molecular mechanisms and therapeutic implications. Neurosurgical focus 28, E8.

17. Angelo, C., Cianchini, G., Grosso, M.G., Zambruno, G., Cavalieri, R., and Paradisi, M. (2001). Association of piebaldism and neurofibromatosis type 1 in a girl. Pediatric dermatology 18, 490-493.
18. Bollag, G., Clapp, D.W., Shih, S., Adler, F., Zhang, Y.Y., Thompson, P., Lange, B.J., Freedman, M.H., McCormick, F., Jacks, T., et al. (1996). Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nature genetics 12, 144-148.
19. Xu, J., Ismat, F.A., Wang, T., Yang, J., and Epstein, J.A. (2007). NF1 regulates a Ras-dependent vascular smooth muscle proliferative injury response. Circulation 116, 2148-2156.
20. Guha, A. (1998). Ras activation in astrocytomas and neurofibromas. The Canadian journal of neurological sciences Le journal canadien des sciences neurologiques 25, 267-281.
21. Parekh, R.B., Dwek, R.A., Rudd, P.M., Thomas, J.R., Rademacher, T.W., Warren, T., Wun, T.C., Hebert, B., Reitz, B., Palmier, M., et al. (1989). N-glycosylation and in vitro enzymatic activity of human recombinant tissue plasminogen activator expressed in Chinese hamster ovary cells and a murine cell line. Biochemistry 28, 7670-7679.
22. Stanley, P. (2011). Golgi glycosylation. Cold Spring Harbor perspectives in biology 3.
23. Garte, S.J., Currie, D.D., and Troll, W. (1987). Inhibition of H-ras oncogene transformation of NIH3T3 cells by protease inhibitors. Cancer research 47, 3159-3162.
24. Voskas, D., Kim, M., and Hurta, R.A. (2001). Platelet-derived growth factor mediated altered expression and regulation of ornithine decarboxylase in H-ras-transformed cell lines. Cellular signalling 13, 401-409.
25. Dennis, J.W., and Laferte, S. (1989). Oncodevelopmental expression of--GlcNAc beta 1-6Man alpha 1-6Man beta 1--branched asparagine-linked oligosaccharides in murine tissues and human breast carcinomas. Cancer research 49, 945-950.
26. Spearman, M.A., Damen, J.E., Kolodka, T., Greenberg, A.H., Jamieson, J.C., and Wright, J.A. (1991). Differential effects of glycoprotein processing inhibition on experimental metastasis formation by T24-H-ras transformed fibroblasts. Cancer letters 57, 7-13.
27. Weber, C.K., Sommer, G., Michl, P., Fensterer, H., Weimer, M., Gansauge, F., Leder, G., Adler, G., and Gress, T.M. (2001). Biglycan is overexpressed in pancreatic cancer and induces G1-arrest in pancreatic cancer cell lines. Gastroenterology 121, 657-667.
28. Carroll, S.L., and Stonecypher, M.S. (2005). Tumor suppressor mutations and growth factor signaling in the pathogenesis of NF1-associated peripheral nerve sheath tumors: II. The role of dysregulated growth factor signaling. Journal of neuropathology and experimental neurology 64, 1-9.
29. Mashour, G.A., Ratner, N., Khan, G.A., Wang, H.L., Martuza, R.L., and Kurtz, A. (2001). The angiogenic factor midkine is aberrantly expressed in NF1-deficient Schwann cells and is a mitogen for neurofibroma-derived cells. Oncogene 20, 97-105.
30. Ling, B.C., Wu, J., Miller, S.J., Monk, K.R., Shamekh, R., Rizvi, T.A., Decourten-Myers, G., Vogel, K.S., DeClue, J.E., and Ratner, N. (2005). Role for the epidermal growth factor receptor in neurofibromatosis-related peripheral nerve tumorigenesis. Cancer cell 7, 65-75.
31. Garratt, A.N., Voiculescu, O., Topilko, P., Charnay, P., and Birchmeier, C. (2000). A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. The Journal of cell biology 148, 1035-1046.
32. Meyer, D., and Birchmeier, C. (1995). Multiple essential functions of neuregulin in development. Nature 378, 386-390.
33. Williams, J.P., Wu, J., Johansson, G., Rizvi, T.A., Miller, S.C., Geiger, H., Malik, P., Li, W., Mukouyama, Y.S., Cancelas, J.A., et al. (2008). Nf1 mutation expands an EGFR-dependent peripheral nerve progenitor that confers neurofibroma tumorigenic potential. Cell stem cell 3, 658-669.

**34. Petrelli, A., and Giordano, S. (2008). From single- to multi-target drugs in cancer therapy: when aspecificity becomes an advantage. Current medicinal chemistry 15, 422-432.**
**35. Rosenbaum, T., Kim, H.A., Boissy, Y.L., Ling, B., and Ratner, N. (1999). Neurofibromin, the neurofibromatosis type 1 Ras-GAP, is required for appropriate P0 expression and myelination. Annals of the New York Academy of Sciences 883, 203-214.**
**36. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G.F. (2003). Met, metastasis, motility and more. Nature reviews Molecular cell biology 4, 915-925.**
**37. Cichowski, K., Shih, T.S., Schmitt, E., Santiago, S., Reilly, K., McLaughlin, M.E., Bronson, R.T., and Jacks, T. (1999). Mouse models of tumor development in neurofibromatosis type 1. Science 286, 2172-2176.**
**38. Evans, D.G., O'Hara, C., Wilding, A., Ingham, S.L., Howard, E., Dawson, J., Moran, A., Scott-Kitching, V., Holt, F., and Huson, S.M. (2011). Mortality in neurofibromatosis 1: in North West England: an assessment of actuarial survival in a region of the UK since 1989. European journal of human genetics : EJHG 19, 1187-1191.**

**Appendices……**
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